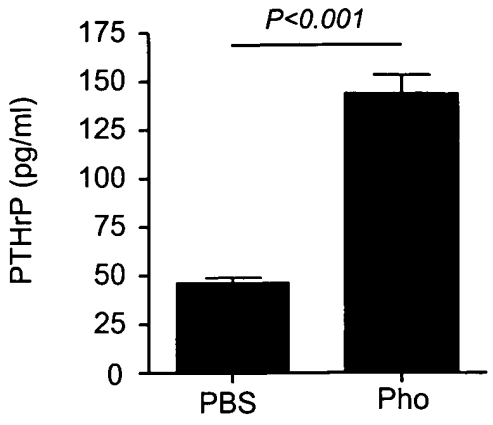
***In Vitro* Experimental Procedure**

Stock cultures of UMR-106 osteoblast cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/liter), penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (2 mM), and sodium pyruvate (1 mM) in a water-saturated atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were passaged every 3–4 days.

To study the effect of phosphoramidon on PTHrP expression, 2 x 10^5 cells were plated in 60-mm tissue culture dishes in 2 ml DMEM for 4 days with one change of medium at day 2. At day 4, cells were washed once with Hams' F-12/DMEM (1:1) without FBS and the medium was replaced with DMEM (phenol red free) supplemented as indicated above except that FBS was substituted for 10% stripped FBS and phosphoramidon (10 μM) or vehicle (PBS) were added. After 24 h, the medium was removed and kept at -80°C while cells were washed twice with PBS and immediately processed for RNA extraction using the RNeasy kit (QIAGEN, Mississauga, ON) according to the manufacturer's instructions. Changes in PTHrP mRNA levels were determined by semiquantitative RT-PCR. Total RNA (2.5–5.0 μg) was reverse-transcribed with oligo$(dT)_{12-18}$ and cDNA amplified by PCR. For cDNA amplification the following sets of specific primers were used: forward: 5'-GCTACTGCATGAC-AAGGGCAAGTCC and reverse 5'-CATCACCCACAGGCTAGCGCCAACT. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as internal control. PTHrP levels in the medium were measured using an immunoradiometric assay (Diagnostic Systems Laboratories, Inc., TX).

Results

Fig. 1



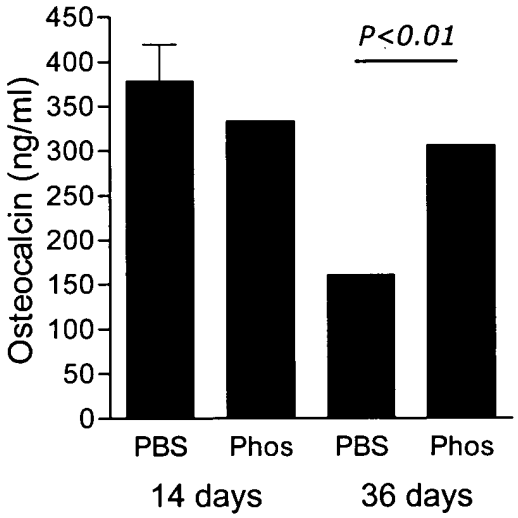
PTHrP mRNA levels were not significantly different in cells treated with phosphoramidon compared to vehicle (PBS)-treated cells (results not shown). However, as illustrated in Fig. 1 immunoreactive PTHrP in medium conditioned by UMR-106 osteoblast cells was significantly increased nearly 3-fold (*P<0.001*, n=5) when cells when cultured in the presence of phosphoramidon (10 μM) compared to vehicle-treated cells. These findings indicate that inhibition of neutral endopeptidases such as NEP, ECE, and PHEX (previously known as PEX) by this general potent inhibitor leads to an increase in secreted PTHrP levels that do not arise from increased PTHrP gene transcription but are likely a consequence of changes in posttranslational processing of the protein because of inhibition of osteoblast endopeptidase activity. Since studies have shown that PTH and PTHrP are not substrates for NEP or ECE, the observed increase in PTHrP levels in the tissue culture medium is likely due to inhibition of PHEX enzymatic activity. PTHrP is a potent endogenous bone anabolic agent. Therefore, the observed rise in PTHrP levels in the osteoblast microenvironment may lead to increased bone formation. This hypothesis was subsequently tested *in vivo*.

***In Vivo* Experimental Procedure**

Eight C57BL/6 one-month-old male mice were purchased from Charles River and housed at controlled temperature and humidity with free access to food (regular chow) and water. Following one week of acclimatization, animals were divided in two groups of 4 animals each and injected intraperitoneally daily with either phosphate-buffered saline (PBS; control group) or PBS with phosphoramidon (Sigma; 200 μg/day; phosphoramidon-treated group). Serum osteocalcin levels were determined by ELISA using Rat-Mid Osteocalcin kit manufactured by Osteometer BioTech A/S (Herlev, Denmark) following 14 and 36 days of treatment.

Results

Fig.2



As shown in Fig. 2 serum osteocalcin levels decreased from 14 to 36 days of treatment with PBS (vehicle, red bars), indicating the expected decrease in bone turnover and specifically, bone formation that normally occurs in mice with increasing age. In sharp contrast, serum osteocalcin levels remained elevated even after 36 days following daily administration of phosphoramidon (200 μg/day, blue bars), and was statistically different than levels in vehicle-treated animals ($P<0.01$). These findings suggest that inhibition of neutral endopeptidases such as NEP, ECE, and PHEX by this general potent inhibitor and the associated increase in PTHrP levels within the skeletal microenviroment leads to an increase in serum osteocalcin level, a marker of bone formation. Therefore inhibition of endopeptidase activity such as PHEX on osteoblasts offers a viable and novel therapeutic

strategy in the treatment of metabolic bone disease such as osteoporosis as was predicted earlier by the inventors of the present patent application. Conversly, **activation** of PHEX enzymatic activity could be used to **decrease** local levels of PTH and PTHrP within the skeletal microenvironment and this could provide a viable therapeutic option in conditions such as fibrous dysplasia and osteitis fibrosa cystica in renal osteodystrophy (very frequently seen in patients with chronic renal failure), that are associated with increased PTH and PTHrP activity.